Award Number: W81XWH-12-1-0624

TITLE: Toward an Inexpensive Test for Vitamin D levels in Blood

PRINCIPAL INVESTIGATOR: Alan C. West

CONTRACTING ORGANIZATION: The Trustees of Columbia University in the City of New York

New York, NY 10027-6900

REPORT DATE: October 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2013	Annual	28September2012-27September2013

4. TITLE AND SUBTITLE
Toward an Inexpensive Test for Vitamin D levels in Blood

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-12-1-0624

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Alan C. West, Scott Banta, Linda Linday, Sudipta Majumdar, Harun F. Ozbakir

E-Mail: acw7@columbia.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The Trustees of Columbia University in the City of New York
2960 Broadway 211 Low Library
New York, NY 10027-6900

8. PERFORMING ORGANIZATION REPORT

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT The objective of the research is to test the feasibility of a vitamin D biosensor based on a selective redox enzyme immobilized onto an electrode. In long-term, this will make it possible to create a technology akin to commercial glucose sensors for in-clinic use as part of routine examinations by both reducing the cost of testing vitamin D levels and reducing the time for testing to less than a few minutes. For this purpose, a synthetic gene for recombinant human CYP27B1 (the enzyme which is involved in vitamin D metabolism) was designed. The enzyme was expressed in *E. coli* and the activity of this enzyme was verified spectrophotometrically. Finally, a synthetic redox mediator (phenosafranine) was found to be successful in mediating electron transfer between electrode and CYP27B1. This is important for introducing a way to electrochemically measure 25(OH)D (circulating form of vitamin D in blood) levels.

15. SUBJECT TERMS
Cytochrome P450, mediated electrochemistry, vitamin D, 25(OH)D, electrochemical biosensors.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	13	

Table of Contents

Introduction

Vitamin D is crucial for the human body due to its role in calcium and bone metabolism. In addition, low blood levels of vitamin D levels have been associated with neuropsychiatric disorders.[1] Vitamin D blood tests, however, are expensive and the results are delayed. The purpose of this research is to test the feasibility of using an electrode modified with an enzyme that hydrolyzes circulating 25-hydroxyvitamin D (25(OH)D) into its biologically active form.[2] This, in the long-term, will make it possible to develop a clinical vitamin D sensor (similar to a commercial glucose sensor) that will reduce both the time and cost for testing. For this purpose, a synthetic gene for the expression of recombinant human cytochrome P450 (CYP27B1) in *E. coli* was designed, and solubilization strategies were developed to enable the purification of the active enzyme. We then developed and characterized the activity of the enzyme using non-natural redox mediators. This work provides a path forward for developing this enzyme into a biosensor for Vitamin D detection.

Body

1. DESIGN, EXPRESSION AND CHARACTERIZATION OF HUMAN CYP27B1, ADX AND ADR ENZYMES

1.1. Cloning, Expression and Purification of Human CYP27B1

Human CYP27B1 gene was codon optimized for expression and purification from *E. coli.* The N-terminal of CYP27B1 gene contains a 32-residue-long mitochondrial targeting sequence, which is truncated from the mature protein.[3] The mitochondrial targeting sequence was removed and Ser-32 was mutated to Met which serves as the initiation codon. The gene was cloned into two different vectors pMAL-c4e (with and without maltose binding protein (MBP)) as a fusion to enhance expression, and pET20b with C-terminal *His-tag* (Figure 1).

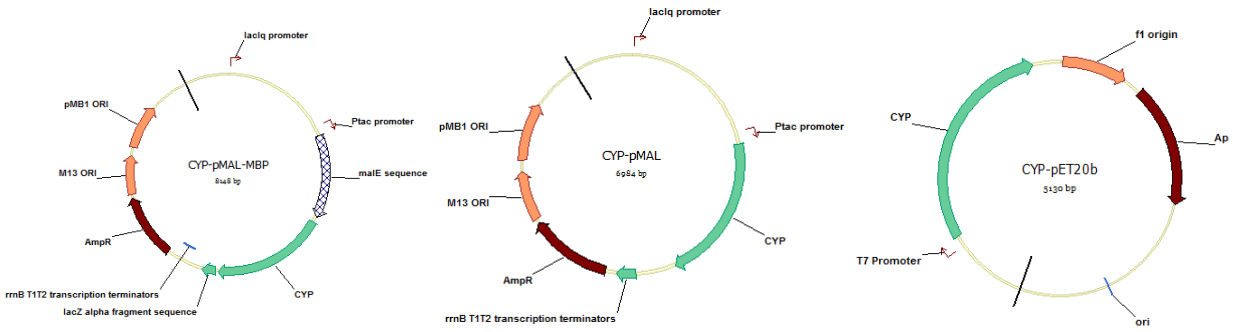

Figure 1: Plasmids containing the CYP27B1 gene. CYP-pMAL-MBP, CYP27B1 cloned in pMAL-c4e vector with MBP; CYP-PMAL, CYP27B1 cloned in pMAL-c4e vector without MBP; and CYP-pET20b, CYP27B1 cloned in pET20b vector.

All three plasmids were transformed into *E. coli* BL21 cells and expressed in the presence of the pGro7 plasmid encoding chaperons GroEL/ES.[4] After protein expression screening, it was found that soluble protein was only obtained from CYP-pET20b. Both CYP-pMAL and CYP-pMAL-MBP enabled expression of CYP27B1, but the expressed protein was predominantly sent to inclusion bodies (Figure 2).

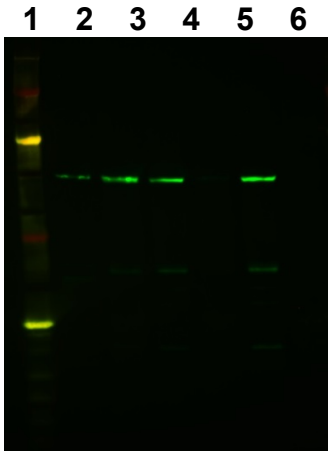

Figure 2: Western blot of CYP27B1 expression screening. Lane 1, 3 and 5 are CYP27B1 inclusion bodies from CYP-pET20b, CYP-pMAL and CYP-pMAL-MBP, respectively. Lane 2, 4 and 6 are CYP27B1 in solution from CYP-pET20b, CYP-pMAL and CYP-pMAL-MBP, respectively.

The cells containing both CYP-pET20b and pGro7 were inoculated in 5 mL TB broth containing 100 µg/mL ampicillin and 35 µg/mL chloramphenicol and incubated overnight at 37 C with shaking. The overnight culture was diluted into 2 x 1 L TB broth containing 100 µg/mL ampicillin and 35 µg/mL chloramphenicol. The cultures were incubated at 37 C until the cell density (OD_{600}) reached about 0.6. At this point, the induction of CYP27B1 and GroEL/ES transcription under the T7 promoter and the araB promoter were initiated by addition of IPTG and arabinose at final concentration of 0.5 mM and 200 µg/mL, respectively. δ-Aminolevulinic acid (ALA) was also supplemented at a final concentration of 1 mM. The cultures were incubated for another 20 h for the expression of CYP27B1 at 28 C.

The recombinant *E. coli.* cells were harvested by centrifugation and suspended in buffer A (20 mM Tris-HCl, pH 7.4 containing 1% CHAPS, 200 mM NaCl, 0.1 mM PMSF, and 20% glycerol). Cells were disrupted by sonication in the presence of HALT protease inhibitor for 20 min in the presence of ice. Cell debris was removed by centrifugation and the resultant supernatant was applied to a Ni affinity column equilibrated with buffer A. The column was washed with 5 column volumes of buffer A. CYP27B1 was eluted with 100 mM imidazole in buffer A (buffer B). Eluted fractions were checked for purity (Figure 3), and the pure fractions were collected and dialyzed in buffer C (50 mM Tris-HCl, pH 7.4 containing 0.1% CHAPS, 200 mM NaCl, 0.1 mM PMSF, and 20% glycerol) and stored at -80 C.

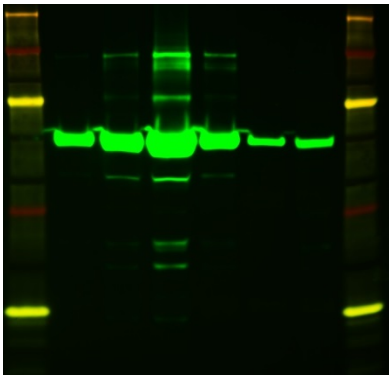

Figure 3: Western blot of pure CYP27B1 fractions.

1.2. Cloning, Expression and Purification of Human Adrenodoxin (Adx) and NADPH-Adrenodoxin Reductase (Adr)

Human adrenodoxin (Adx), and NADPH-adrenodoxin reductase (Adr) genes were amplified from the pTC27A1 plasmid (Figure 4).[5]

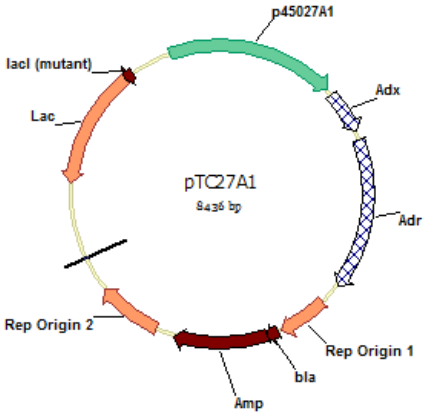

Figure 4: Vector pTC27A1 containing human Adx, Adr and p45027A1.

Adx and Adr were cloned into pMAL-c4E separately, creating pMAL-ADX (Figure 5) and pMAL-ADR (Figure 6), respectively. Both Adx and Adr were also cloned together with CYP27B1, creating pCXR (Figure 7).

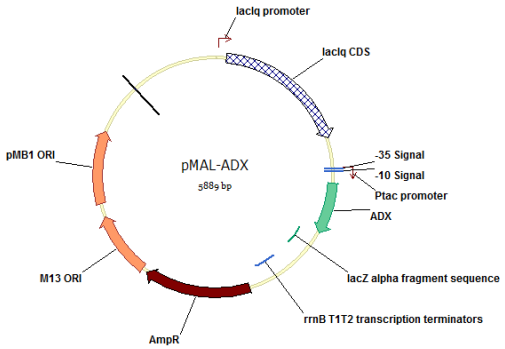

Figure 5: Vector pMAL-ADX containing human Adx.

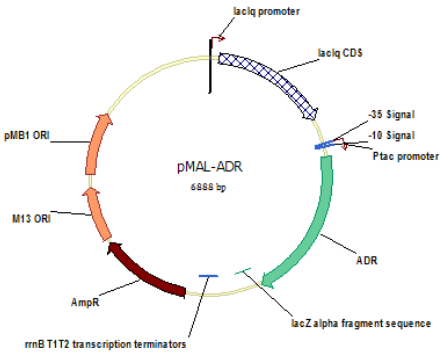

Figure 6: Vector pMAL-ADR containing human Adr.

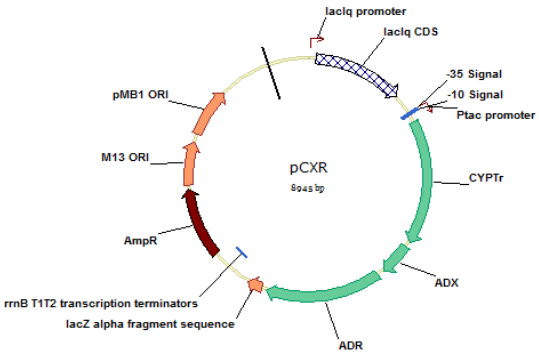

Figure 7: Vector pCXR containing CYPTr, Adx and Adr.

All three plasmids, pMAL-ADX, pMAL-ADR and pCXR, were transformed into *E. Coli* BL21 and BL21/pGro7 competent cells and grown in 5 mL TB media supplemented with 0.4% glycerol (v/v), 2 mg/mL bactopeptone, 1 mM NaCl, 1mM thiamine, a mixture of trace elements (50 µM ferric citrate, 1mM $MgCl_2$, 2.5 mM $(NH_4)_2SO_4$), 100 µg of Amp/mL and 35 µg of Cam/mL at 37 C till OD_{600} ~ 0.6 and were induced with 1 mM IPTG, 1 mM δ-aminolevulinic acid (ALA), 200 µg of Arabinose/mL, 2µg of Hemin/mL and grown at 28 C for ~ 24 h. Cells were harvested with centrifugation, suspended in 0.5 mL Tris-HCl buffer, pH 7.4 and sonicated. Both cell debris and supernatants were subjected to SDS-PAGE and western blotting was performed to determine protein expression (Figure 8).

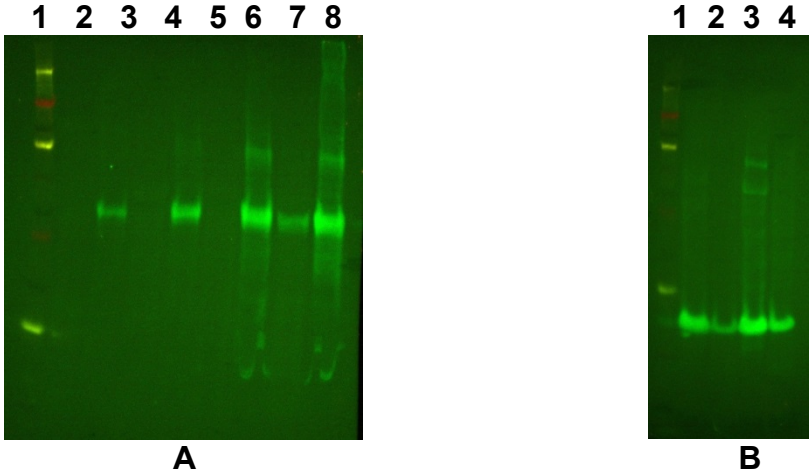

Figure 8: A) 1. pCXR/BL21 in solution, 2. pCXR/BL21 in insoluble fractions, 3. pCXR/BL21/pGro7 in solution, 4. pCXR/BL21/pGro7 in insoluble fraction, 5. Adr/BL21 in solution, 6. Adr/ BL21 in insoluble fraction, 7. Adr/BL21/pGro7 in solution, 8. Adr/BL21/pGro7 in insoluble fraction. B) 1. Adx/BL21 in solution, 2. Adx/BL21 in insoluble fraction, 3. Adx/BL21/pGro7 in solution, and 4. Adx/BL21/pGro7 in insoluble fraction.

All three plasmids, pCXR, pMAL-Adr and pMAL-Adx, were found to enable protein expression, but in the case of pCXR the proteins were found in inclusion bodies. Therefore, we decided to purify Adr and Adx separately from BL21/pGRO7 cells.

2 L cultures of pMAL-Adr/BL21/pGro7 and pMAL-Adx/BL21/pGro7 were grown in the above mentioned media. Cells were harvested with centrifugation, suspended in 40 mL buffer D (100 mM Tris-HCl, pH 7.4 containing 1% CHAPS, 500 mM NaCl, 0.5 mM EDTA, 0.1 mM DTT, 0.1 mM PMSF, and 20% glycerol) and disrupted by sonication (20 min, 5 sec On, 10 sec Off). Cell debris was removed by centrifugation, and supernatant was loaded into pre-equilibrated Ni column with buffer D and eluted with 100 mM imidazole in buffer D (buffer E). The fractions were run in SDS-PAGE gel and pure fractions were collected for dialysis into buffer C.

1.3. Spectrophotometric activity assay for CYP27B1

The hydroxylation of 25(OH)D to 1,25$(OH)_2$D is catalyzed by CYP27B1 in the presence of NADPH as a cofactor. NADPH is oxidized to NADP by transferring electrons to CYP27B1 via Adx and Adr for required hydroxylation.[6-8] So, the rate of 25(OH)D hydroxylation by CYP27B1 can be monitored spectrophotometrically by monitoring the rate of NADPH oxidation to NADP at 340 nm.

Pure CYP27B1 was used for the activity assay at room temperature in 50 mM Tris-HCl buffer, pH 7.4 containing 0.1 mM EDTA, 0.1% CHAPS and 0.2% glycerol. The reactions were followed by the oxidation of 192 µM NADPH, in the presence of 2 µM Adx, 0.3 µM Adr, 30 µM 25(OH)D and 125 µM CYP27B1. As a control, NADPH oxidation was monitored in absence of CYP27B1, where 25(OH)D, Adx and Adr were added to the reaction mixture. No NADPH oxidation was observed (Figure 9, green line); confirming

NADPH oxidation is independent of Adx and Adr. NADPH oxidation was, also, monitored in the absence of 25(OH)D, where Adx, Adr and CYP27B1 were added to the reaction mixture. NADPH oxidation was observed independent of 25(OH)D addition (Figure 9, red line). This is due to the formation of superoxide or peroxide independent of substrate during the electron transfer from NADPH to CYP27B1, resulting in substrate independent NADPH oxidation. When substrate was added to the reaction, the initial rate of NADPH oxidation increased two-fold (Figure 9, blue line). Therefore the rate of 25(OH)D hydroxylation by CYP27B1, 0.8 min^{-1}, was calculated by subtracting the background rate of NADPH oxidation in the absence of 25(OH)D. It should be noted that the substrate-independent activity provides very strong incentive to pursue non-natural redox mediators, discussed below.

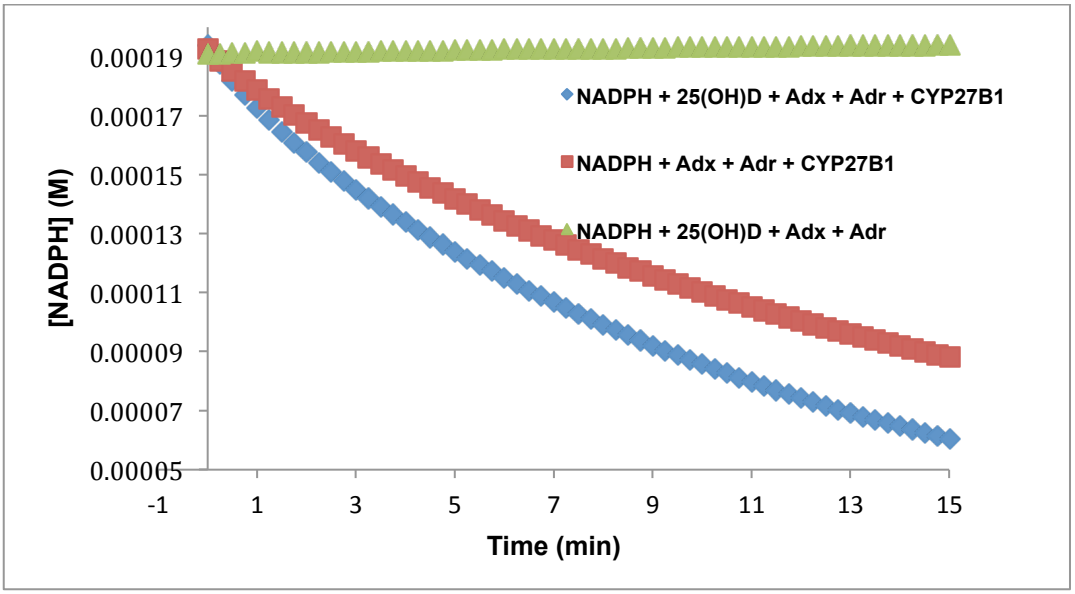

Figure 9: CYP27B1 activity assay results. There is significant NADPH oxidation in the absence of 25(OH)D, however the NADPH oxidation increases with the presence of 25(OH)D indicating the presence of active enzyme.

2. ELECTROCHEMICAL MEASUREMENT OF 25(OH)D LEVELS

2.1. Investigation of Mediated Electron Transfer

Mediated electron transfer using synthetic redox mediators is an alternative to the natural electron transfer systems in P450s, which use NADPH as electron supplier and Adx and Adr as electron transfer enzymes. Synthetic redox mediators transfer electrons rapidly from the electrode to the active redox site of the enzyme.

We have explored two synthetic redox mediators, phenosafranine (PSF) and safranin T (SAF); further we have recently synthesized a third redox mediator, but it has not yet been characterized. These organic mediators contain two amine groups that can interact with hydrophilic residues of enzyme and these residues may play an important role in electron transfer by creating a docking surface .[9] In Figure 10, the oxidation potential of PSF has been investigated with cyclic voltammetry, using Au as the working electrode, Ag/AgCl as

the reference electrode and Pt as the counter electrode. It has been found that PSF has an oxidation potential of -0.242 V (vs. SHE) and this value is close to the literature value of -0.252 V (vs. SHE).[10]

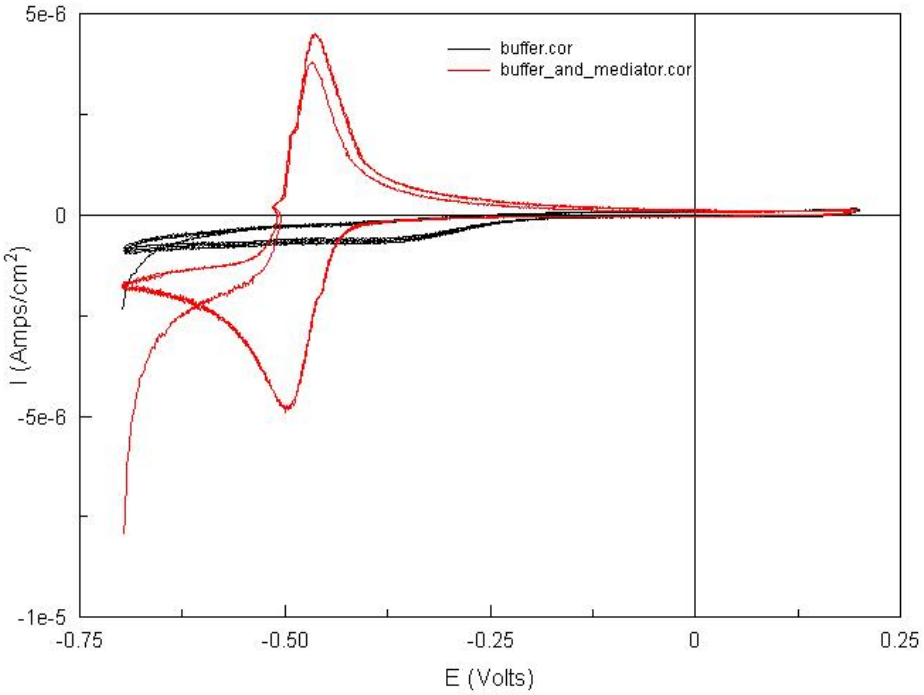

Figure 10: Cyclic voltammetry (CV) for the characterization of the oxidation potential of PSF. The black curve shows the CV for the electrolyte solution (0.1 M PBS, pH 7.4), whereas the red curve represents the case where 0.6 mM PSF is solubilized in the same electrolyte solution. Potential values are with respect to the Ag/AgCl reference electrode.

Following this experiment, varying amounts of CYP and the substrate (25(OH)D) were added to the PSF containing solutions in order to observe if mediation occurs. In this case, the enzyme, mediator and substrate are in solution, meaning the system is homogenous. In homogeneous mediation, the mediator first changes its redox state at the electrode. This mediator diffuses from the electrode and transfers electrons to the enzyme, and in turn, this enzyme reacts with the substrate to produce the desired product.[11]

Cyclic voltammetry was utilized to observe the changes in the oxidation/reduction peaks. Figure 11 shows the case where substrate (25(OH)D) concentration is kept constant at 5 μM, and varying amounts of CYP27B1 (0-10 μM) are added into the solution, and a change in the peak current is plotted as a function of concentration of CYP27B1. When the amount of enzyme in the system was increased, the peak where PSF oxidation/reduction peaks are observed is altered; meaning the amount of mediator oxidized/reduced is changed. This is in agreement with the proposed homogeneous mediation scheme. Similar results can be seen in Figure 12, where this time the CYP27B1 concentration is kept constant and 25(OH)D concentration is changed. In Figure 12, the change in the current for different modes of operation (oxidation vs reduction peaks) is plotted as a function of

the concentration of 25(OH)D.

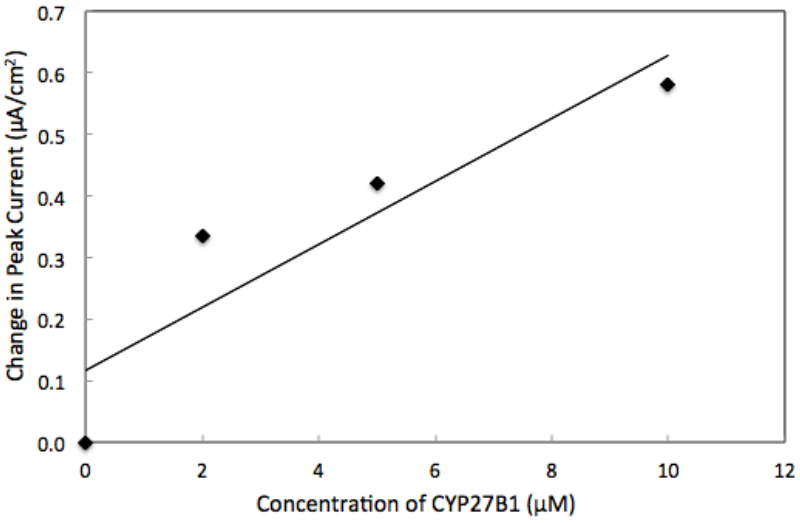

Figure 11: Change in peak currents as a function of concentration of CYP27B1. Experiments were made in the presence of 2000 ng/mL 25(OH)D. The electrolyte solution is 0.1 M PBS (pH 7.4) that contains 0.3 mM mediator (phenosafranine).

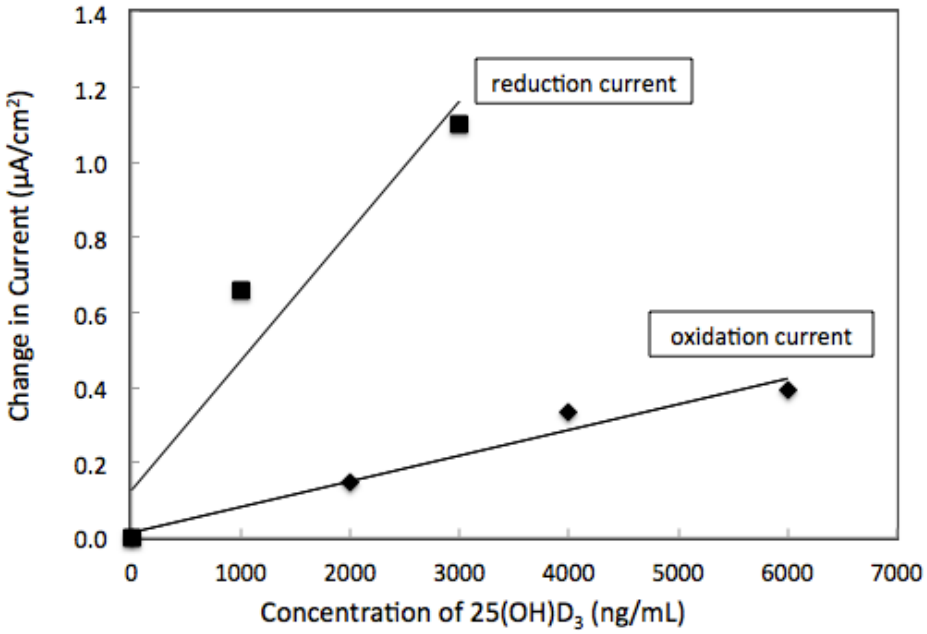

Figure 12: Change in reduction and oxidation currents as a function of concentration of 25(OH)D_3. Reduction current experiments were made in the presence of 2 µM CYP27B1 whereas oxidation experiments were made in the presence of 5 µM CYP27B1. The electrolyte solution is 0.1 M PBS (pH 7.4) that contains 0.3 mM mediator (phenosafranine).

Similar experiments were also performed for the other mediator, safranin T. In control experiments, where only mediator and substrate added (in order to see if the mediator reacts with the substrate), it has been observed that safranin T reacts directly with the substrate, limiting its practical utility. In contrast, the PSF does not react directly with the substrate 25(OH)D.

Key Research Accomplishments

1. A synthetic gene for recombinant human CYP27B1 was designed for *E. coli*.
2. CYP27B1 was expressed and purified.
3. Recombinant bovine ferredoxin (ADX) and ferredoxin reductase (ADR) were expressed and purified.
4. The activity of CYP27B1 was verified via spectrophotometric NADPH oxidation assay.
5. Phenosafranine, a synthetic redox mediator, has been found to be successful in mediating electron transfer between electrode and CYP27B1.

Reportable Outcomes

1. Manuscript to be submitted upon completion of the additional experiments
2. Proposal to be submitted based on the results obtained in this report

Conclusions

In this research, the feasibility of a vitamin D biosensor based on a selective redox enzyme immobilized onto an electrode was investigated. A synthetic recombinant enzyme, CYP27B1, was expressed in *E. coli*. This enzyme is responsible for hydroxylation of 25-hydroxyvitamin D (25(OH)D, circulating form of vitamin D in blood) into its active form. This enzyme is membrane associated, and therefore solubilization strategies were developed. The activity of the enzyme was verified spectrophotometrically using NADPH oxidation assay. For this assay, recombinant bovine ferredoxin (ADX) and ferredoxin reductase (ADR) enzymes were expressed and purified. Finally, a method was established for electrochemical measurement of 25(OH)D levels by introducing a non-natural redox mediator. Two synthetic redox mediators in solution with enzyme were explored and phenosafranine is found to be capable of mediating electron transfer from an electrode to the enzymatic active site, suggesting we can detect the activity of the enzyme electrochemically. For future work, different strategies need to be explored for immobilization of the enzyme and the redox mediator to lower the reagents needed for activity measurements and to obtain a better detection signal. These promising results chart a path forward for the development of a vitamin D biosensor akin to commercial glucose sensor that will reduce both the time for testing and the cost of 25(OH)D assay and that can be utilized clinically.

References

(1) Holick, M. F. *The New England journal of medicine* **2007**, *357*, 266.
(2) Rhieu, S. Y.; Ludwig, D. R.; Siu, V. S.; Palmore, G. T. R. *Electrochemistry Communications* **2009**, *11*, 1857.
(3) Annalora, A. J.; Goodin, D. B.; Hong, W.-X.; Zhang, Q.; Johnson, E. F.; Stout, C. D. *Journal of Molecular Biology* **2010**, *396*, 441.
(4) Uchida, E.; Kagawa, N.; Sakaki, T.; Urushino, N.; Sawada, N.; Kamakura, M.; Ohta, M.; Kato, S.; Inouye, K. *Biochemical and Biophysical Research Communications* **2004**, *323*, 505.
(5) Salamanca-Pinzon, S. G.; Guengerich, F. P. *Protein Expr Purif* **2011**, *79*, 231.
(6) Hlavica, P. *Biotechnology Advances* **2009**, *27*, 103.
(7) Chefson, A.; Auclair, K. *Molecular bioSystems* **2006**, *2*, 462.
(8) Kumar, S. *Expert opinion on drug metabolism & toxicology* **2010**, *6*, 115.
(9) Çekiç, S. Z.; Holtmann, D.; Güven, G.; Mangold, K.-M.; Schwaneberg, U.; Schrader, J. *Electrochemistry Communications* **2010**, *12*, 1547.
(10) Fultz, M. L.; Durst, R. A. *Analytica Chimica Acta* **1982**, *140*, 1.
(11) Chaubey, A.; Malhotra, B. D. *Biosensors and Bioelectronics* **2002**, *17*, 441.